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Relationship between Secondary Metabolism and Fungal Development

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INTRODUCTION

Fungi are remarkable organisms that readily produce a wide range of natural products often called secondary metabolites. In this review we will use the terms interchangeably. In many cases, the benefit these compounds confer on the organism is unknown (21). However, interest in these compounds is considerable, as many natural products are of medical, industrial and/or agricultural importance. Some natural products are deleterious (e.g., mycotoxins), while others are beneficial (e.g.,

antibiotics) to humankind (35). Although it has long been noted that biosynthesis of natural products is usually associated with cell differentiation or development, and in fact most secondary metabolites are produced by organisms that exhibit filamentous growth and have a relatively complex morphology, until recently the mechanism of this connection was not clear. A critical advance in this regard was the establishment of a G-protein-mediated growth pathway in *Aspergillus nidulans* that regulates both asexual sporulation and natural product biosynthesis (55). Since then, several studies have provided insight into other molecules and pathways that link chemical and morphological differentiation processes in fungi. The focus of this review is to provide an overview of the research establishing this connection, and this review covers the progress made to date in elucidating relationships between natural

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TABLE 1. Fungal secondary metabolites associated with sporulation processes and development

Secondary metabolite	Producing fungus	Association with development	Reference(s)
Linoleic-acid derived psi factor	<i>Aspergillus nidulans</i>	Induces sporulation; affects ratio of asexual to sexual spore development	23, 26, 27, 79
Zearalenone	<i>Fusarium graminearum</i>	Induces sporulation; enhances perithecial formation	125
Butyrolactone I	<i>Aspergillus terreus</i>	Induces sporulation and lovastatin production	102
Melanin	<i>Colletotrichum lagenarium</i>	Associated with appressorial formation	116
Melanin	<i>Alternaria alternata</i>	UV protection of spore	63
Melanin	<i>Cochliobolus heterotrophus</i>	Required for spore survival	72
Spore pigment	<i>Aspergillus fumigatus</i>	Required for virulence	121
Mycotoxins	<i>Aspergillus</i> spp.	Produced after sporulation	12, 49, 98
Patulin	<i>Penicillium urticae</i>	Antibiotic; produced after sporulation	103

product metabolism and fungal development. We conclude with speculation as to why such a relationship could be of value to the organism.

SECONDARY METABOLISM IS ASSOCIATED WITH DEVELOPMENTAL PROCESSES

Secondary metabolism is commonly associated with sporulation processes in microorganisms (56, 77, 113), including fungi (21, 103). Secondary metabolites associated with sporulation can be placed into three broad categories: (i) metabolites that activate sporulation (for example, the linoleic acid-derived compounds produced by *A. nidulans* [23, 26, 27, 79]), (ii) pigments required for sporulation structures (for example melanins required for the formation or integrity of both sexual and asexual spores and overwintering bodies [6, 63]), and (iii) toxic metabolites secreted by growing colonies at the approximate time of sporulation (for example the biosynthesis of some deleterious natural products, such as mycotoxins [55, 120]). These and other examples of secondary metabolites that fit into these categories are shown in Table 1.

Secondary Metabolites That Are Sporogenic Factors

Secondary metabolite production usually commences late in the growth of the microbe, often upon entering the stationary or resting phase (21). In early observations it was noted that the environmental conditions required for sporulation and secondary metabolism were often similar and were more stringent than those for pure vegetative growth (21, 51, 103). Although it was once thought that natural products were essential for sporulation, there are many examples of fungal strains that still sporulate but are deficient in secondary metabolite production, for example, *Penicillium urticae* patulin mutants (103) and *A. nidulans* sterigmatocystin mutants (106). In addition, some secondary metabolites such as brevianamides A and B produced by *Penicillium brevicompactum* appear only after conidiation has commenced (14). It is possible, however, that some natural products have subtle effects on sporulation, as recent studies of *A. nidulans* sterigmatocystin mutants indicate that they exhibit a decrease in asexual spore production not detectable by the unaided eye (97, 108).

Some secondary metabolites have easily observable effects on morphological differentiation in fungi. Several studies show that compounds excreted by mycelium can induce asexual and sexual sporulation in other fungi; this phenomenon operates across species and genera (51, 88). In most cases, these compounds have not been identified but are presumed to be natural products produced as the mycelia ages. Other natural products affecting fungal development are better characterized. *Fusarium graminearum* produces an estrogenic mycotoxin called zearalenone that enhances perithecial production in *F. graminearum*. The addition of dichloros, an inhibitor of zearalenone synthesis, inhibits the sexual development of this fungus (125). Butyrolactone I, an inhibitor of eukaryotic cyclin-dependent kinases produced by *Aspergillus terreus*, increases hyphal branching, sporulation, and production of another secondary metabolite, lovastatin, in this fungus (102). Butyrolactone-containing molecules act as self-regulating factors in some bacteria and control many biological functions such as antibiotic and virulence factor production (33). In *A. nidulans*, endogenous oleic acid- and linoleic acid-derived molecules called psi factor govern the ratio of asexual to sexual spores in this organism (23, 26).

Pigments

Some fungal pigments are natural products associated with developmental structures, the most common being melanins. Melanins are generally dark brown pigments formed by oxidative polymerization of phenolic compounds and are synthesized during spore formation for deposition in the cell wall. Melanin biosynthesis has been well studied in pathogenic fungi, where the pigment not only contributes to the survival of the fungal spore by protecting against damaging UV light but is also an important virulence factor.

In *Colletotrichum lagenarium*, melanin biosynthesis has been associated with the formation of appressoria (116). Appressoria are infection structures required for host penetration, and any impairment in their formation can reduce virulence. In *Alternaria alternata*, melanin deposition is also involved in spore development. Disruption of the *A. alternata* melanin biosynthetic gene *brm2* dramatically decreases melanin pro-

duction in this fungus. The conidia produced are reduced in diameter and are more sensitive to UV light than the wild type (63). In another fungus, the maize pathogen *Cochliobolus heterotrophus*, fitness studies of albino spore mutants in a greenhouse indicated that melanin production is required for survival of this fungus (72).

The effect of melanin biosynthesis on the virulence of fungal human pathogens has also been studied. In *Aspergillus fumigatus*, a cause of invasive aspergillosis in immunocompromised patients, spore pigment is a virulence factor. Disruption of the *alb1* gene, which encodes a putative polyketide synthase, creates a pigmentless conidial phenotype and leads to a significant reduction in fungal infection of a murine model (121).

Mycotoxins

The most deleterious of natural products, in terms of health effects, are the mycotoxins. A relationship between mycotoxin production and sporulation has been documented in several mycotoxigenic genera. For example, in *Aspergillus parasiticus* certain chemicals that inhibit sporulation have also been shown to inhibit the production of aflatoxin (98). In *A. parasiticus* and *A. nidulans* chemical inhibition of polyamine biosynthesis inhibits sporulation and aflatoxin and sterigmatocystin production, respectively (49). Several papers have shown that *Aspergillus* mutants deficient in sporulation are also unable to produce aflatoxin (see reference 12 and references therein). In 1977, it was shown that *Claviceps purpurea* mutants lacking the ability to form conidia on agar plates also produce less toxic alkaloids (93). In the same year, Sekiguchi and Gaucher (103) reported that *P. urticae* NRRL 2159A mutants blocked at an early stage of conidiation produced markedly less patulin under growth conditions which normally supported patulin biosynthesis. This study suggests an indirect effect of sporulation on patulin biosynthesis (although patulin biosynthetic mutations have no effect on conidiation [103]). In *Fusarium*, the case for the existence of a genetic connection between sporulation and mycotoxin production has recently been identified. A mutation in the *Fusarium verticillioides* *FCC1* gene results in reduced sporulation and reduced fumonisin B₁ biosynthesis (104). Also, introducing a dominant-activated allele of an *A. nidulans* α G-protein into *Fusarium sporotrichioides* produces strains with elevated production of T-2 mycotoxin but reduced conidium formation (115).

REVIEW OF *ASPERGILLUS* DEVELOPMENT AND MYCOTOXIN PRODUCTION

Aspergillus Developmental Structures

To fully understand the relationship between sporulation and mycotoxin biosynthesis, we will now briefly review these developmental processes in *Aspergillus* with a focus on *A. nidulans*. Most *Aspergillus* spp. propagate solely by asexual spores called conidia (e.g., *Aspergillus flavus* and *A. parasiticus*), while other species produce both conidia and sexual spores called ascospores (e.g., *A. nidulans*, teleomorph: *Emericella nidulans*). *A. nidulans* conidia are formed on specialized complex structures called conidiophores, while ascospores are produced inside spherical sexual fruiting bodies called cleistothecia. Most

asexual species, including *A. flavus* and *A. parasiticus*, form resistant structures called sclerotia. For *Aspergillus* species that are problematic opportunistic pathogens of plants and animals (including humans), conidia serve as the major source of inoculum.

The formation of conidiophores begins with a stalk that extends from a thick-walled foot cell. The tip of the stalk begins to swell, forming a vesicle. From the vesicle, cells called sterigmata are formed (31, 81, 86), and chains of conidia originate from the sterigmata. Genetic and molecular studies of conidial reproduction in *A. nidulans* identified a gene, *brlA* (31), that encodes a transcriptional regulator proposed to govern the activation of developmental genes at the time of vesicle formation (2, 3). Loss-of-function *brlA* mutations result in no conidiation and abnormally long conidiophore stalks (17, 58) and do not affect sterigmatocystin production in *A. nidulans* (49). However, forced expression of *brlA* from an inducible promoter in vegetative cells causes immediate activation of conidiation-specific genes, resulting in conidiophore production (8). The effect of *brlA* overexpression on sterigmatocystin production has not been investigated. Both conidiation and sterigmatocystin biosynthesis are coregulated, at a step prior to *brlA* expression, by a common signal transduction pathway. This regulatory pathway will be the subject of our review in sections below.

Aflatoxin and Sterigmatocystin Biosynthesis

Since the discovery of aflatoxin and its deleterious properties to humans and animals, efforts have been directed toward the understanding of the molecular mechanisms leading to its biosynthesis. The last decade has seen significant progress in characterizing the genes required for aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* and sterigmatocystin biosynthesis in *A. nidulans* (sterigmatocystin is the penultimate precursor in the aflatoxin biosynthetic pathway).

Genes required for the synthesis of aflatoxin and sterigmatocystin are well conserved between aspergilli and are located in large gene clusters (18, 120, 130). The relative order and transcriptional direction of some of the homologous gene pairs though are not conserved (64). Thus far, most of the genes in the respective clusters have been shown to encode enzymes required for toxin biosynthesis (64). At least two genes, *aflR* and *aflJ*, play a regulatory role (80, 91, 131). Several studies of *aflR* have demonstrated it encodes a sterigmatocystin/aflatoxin pathway-specific transcription factor (29, 119, 126, 131). *aflR* deletion mutants in all three *Aspergillus* spp. do not express biosynthetic genes in the sterigmatocystin-aflatoxin cluster nor do they produce sterigmatocystin or aflatoxin (29, 92, 131). Loss of *aflR* does not stop spore or sclerotial production but does affect the numbers of spores (97, 108) and sclerotia (25, 120) formed. The function of *aflJ* is not well defined, but disruption of *aflJ* in *A. flavus* results in a failure to produce aflatoxin (80).

Environmental Factors That Affect Mycotoxin Production and Development in *Aspergillus* spp.

Some environmental factors affect mycotoxin production, conidiation, and cleistothecial and sclerotial production con-

currently. Physical parameters affecting mycotoxin and/or spore production in *Aspergillus* spp. include temperature (42), availability of an air-surface interface (50), and pH (20, 32). Nutritional factors such as carbon source and nitrogen source can also affect both mycotoxin production and morphological differentiation (65). Additionally, some compounds present in seeds commonly infected by *Aspergillus* species can influence both toxin production and fungal development (22, 24, 48, 135).

Effects of pH

Although aflatoxin and sterigmatocystin production, like that of penicillin, appears to be influenced by growth medium pH, studies addressing pH regulation of these mycotoxins have produced complex and at times contradictory results. Cotty (32) determined a link between aflatoxin production and sclerotial morphogenesis based on changes of both chemical and morphological differentiation in response to pH. At pH 4.0 or below, sclerotial production is reduced by 50% in *A. flavus* while aflatoxin production is maximal (32). However, Buchanan and Ayres (20) concluded from their work that the initial pH of growth medium was not an important determinant for mycotoxin production. In fact, the effect of pH on aflatoxin biosynthesis is dependent on the composition of growth media. Keller et al. (65) showed that *A. nidulans* and *A. parasiticus* produced less mycotoxin as the pH of the growth medium increased. In addition, pH-sensing mutants of *A. nidulans* exhibit both sporulation (94, 118) and sterigmatocystin production aberrations (94). Further research is necessary to elucidate the complex interactions between pH and other environmental factors that influence morphological and chemical differentiation in *Aspergillus*.

Effects of Carbon and Nitrogen Source

Availability and type of carbon and nitrogen source affect sterigmatocystin and aflatoxin production. Simple sugars such as glucose, fructose, sucrose, and sorbitol as sole carbon sources support high fungal growth, sporulation, and aflatoxin production (1, 19, 59, 75, 76). In contrast, peptone (1, 19, 42, 75) and the more-complex sugars like galactose, xylose, manitol, and lactose (59) do not support aflatoxin production well.

The choice of nitrogen source used in the growth medium can have different effects on sterigmatocystin and aflatoxin production in different *Aspergillus* species. Nitrate as the nitrogen source has been shown by some groups to repress the synthesis of aflatoxin intermediates in *A. parasiticus* (59, 85) but enhance sterigmatocystin production in *A. nidulans* (42). Feng and Leonard (42) also observed no sterigmatocystin production in ammonium-containing media. Other studies (65, 82) indicate sterigmatocystin and aflatoxin production increases in ammonium-based media and decreases in nitrate-based medium. Nitrogen source influences not only mycotoxin production but also the formation of developmental structures in *Aspergillus* spp. Studies in which *A. flavus* is grown on agar media containing either nitrate or ammonium as the sole nitrogen source have shown that development of sclerotia occurs on nitrate but not on ammonium (13).



FIG. 1. Sporogenic effect of linoleic acid-derived compounds on *Aspergillus*. Conidia of *A. flavus* were spread evenly over YGT medium (26) at a concentration of 10^6 spores/ml. Discs containing 1 mg of 13S-hydroperoxylinoleic acid (L) or solvent control (R) were placed on the surface, and the plate was incubated at 29°C for 2 days. Conidium production is induced by 13S-hydroperoxylinoleic acid (24).

Effects of Host Seed Constituents

In *A. nidulans*, linoleic acid is metabolized into a series of sporogenic molecules called psi factors (27). Calvo et al. (24) have demonstrated that linoleic acid and the hydroperoxylinoleic acid derivatives from seeds increase asexual spore production in *A. flavus*, *A. parasiticus*, and *A. nidulans*. Figure 1 demonstrates how the addition of 13S-hydroperoxylinoleic acid to growth medium can stimulate conidiation of *A. flavus*. The seed fatty acids closely resemble psi factors in structure (Fig. 2). As discussed earlier, psi factors are fatty acid-derived compounds synthesized endogenously by *Aspergillus* (27) that influence the development of this fungus. The results presented above suggest that the sporogenic effect of seed fatty acids could take place through interference and/or mimicking of psi factor.

Linoleic acid also supports aflatoxin production, whereas the hydroperoxylinoleic acid derivatives and downstream breakdown products are reported to inhibit (22, 36, 48, 127) or stimulate (22, 34, 89) aflatoxin production. This differential effect on aflatoxin production might be associated with the placement of functional groups on the carbon backbone of fatty acid derivatives. For example, Burow et al. (22) have shown specifically that treatment of *Aspergillus* with 13S-hydroperoxylinoleic acid decreases the accumulation of aflatoxin biosynthetic gene transcripts. On the other hand, 9S-hydroperoxylinoleic acid lengthens the time aflatoxin and sterigmatocystin transcripts accumulate.

Many other plant compounds have been reported to affect fungal growth and aflatoxin production. For a review of these metabolites, see reference 134.

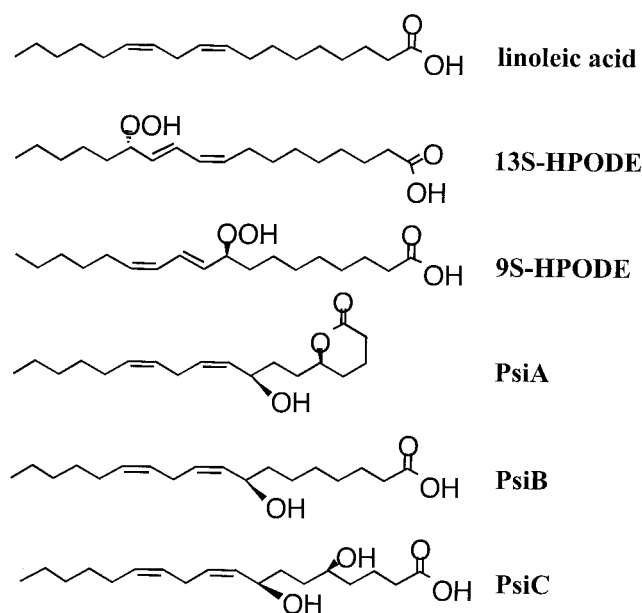


FIG. 2. Structures of linoleic acid and linoleic acid-derived molecules from seed-, 13S-hydroperoxylinoleic acid (13S-HPODE)- and 9S-HPODE-, and linoleic acid-derived sporogenic molecules from *A. nidulans*, psiA, psiB, and psiC.

SIGNAL TRANSDUCTION PATHWAY

The previous section demonstrates that interactions between sporulation and mycotoxin production in relation to environmental factors are not understood at a genetic level. Presumably, the *Aspergillus* fungal cell receives many developmental and mycotoxin-stimulating signals from the environment. The detection and integration of these signals leading to a comprehensive response by the fungus could involve many genetic elements. However, once these signals are transmitted into the fungal cell, it appears that a common signal transduction pathway is partially responsible for tying fungal development to natural product biosynthesis. A major contribution to the elucidation of this genetic connection between fungal development and biosynthesis of natural products was made in the study of *Aspergillus* developmental mutants first described by Bennett and collaborators (10–12), in which they observed that conidial mutants also lost the ability to produce aflatoxin. Further studies by Kale et al. (60, 61) revealed that these *A. flavus* and *A. parasiticus* developmental mutants also lack *aflR* and aflatoxin enzymatic gene transcripts and fail to accumulate other aflatoxin pathway intermediates. A similar phenotype was found in developmental *A. nidulans* mutants called “fluffy” (37, 78, 117, 123). Hicks et al. (55) demonstrated that some of these *A. nidulans* developmental mutants are also deficient in sterigmatocystin production. These findings led to the discovery of a signal transduction pathway regulating both conidiation and sterigmatocystin-aflatoxin biosynthesis.

Overview of Signal Transduction in Fungi

In *Saccharomyces cerevisiae*, several signaling pathways operate to regulate filamentous growth and differentiation (71). One prominent pathway involves components of the mitogen-

activated protein (MAP) kinase pathway that is activated in response to pheromones and nutrition availability. In haploid *S. cerevisiae* cells, the MAP kinase pathway mediates the response to pheromones during mating (71). In diploid cells, the same signaling pathway is used to stimulate filamentous growth in response to nitrogen limitation and other environmental signals (46). A second well-studied signal transduction pathway in *S. cerevisiae* operates through nutrient sensing and functions in parallel to the MAP kinase pathway. It involves a heterotrimeric G-protein-coupled receptor, adenylate cyclase, cyclic AMP (cAMP) signaling molecules, and protein kinase A (PKA) and regulates pseudohyphal growth (71). Upstream of PKA are at least two other small, monomeric GTP-binding proteins called Ras1 and Ras2 (71). Ras1 and Ras2 regulate cAMP production by interacting with adenylate cyclase, and Ras2 regulates the MAP kinase pathway during filamentous growth. In *Schizosaccharomyces pombe*, a Ras1 homolog plays a role in MAP kinase pathway activation but not in regulating cAMP production (71).

Some proteins acting downstream of PKA in the G-protein signaling pathway also interact with components of the MAP kinase pathway (66). For example, MAP kinase and cAMP signaling pathways both activate expression of the *FLO11* gene. This gene encodes a cell surface protein required for pseudohyphal formation in *S. cerevisiae* (101). Other studies of *S. cerevisiae* indicate that cAMP-dependent protein kinases might positively regulate signaling in the MAP kinase cascade (83).

Protein kinase signaling pathways have also been examined in some pathogenic fungi (see reference 71 for a recent review). Briefly, in *Ustilago maydis*, cAMP signaling appears to control the switch between budding and filamentous growth. This switch is essential for the ability of the fungus to cause diseases of crops (47). cAMP signaling is also involved in appressorial formation in *Magnaporthe grisea*, a key step in the infection process of rice (see reference 66 and references therein). An α or β G-protein subunit of the human pathogen *Cryptococcus neoformans* regulates mating, virulence, and melanin biosynthesis (6). From these examples it is clear that the G-protein signaling pathway is essential for fungal growth and infection.

A G-Protein/cAMP/PKA Signaling Pathway Connects Sporulation and Sterigmatocystin-Aflatoxin Production in *Aspergillus* spp.

Characterization of fluffy mutants in *Aspergillus* G-protein signaling. Insight into an *Aspergillus* G-protein signal transduction pathway originated by complementation of single-locus asexual developmental mutants called fluffy. Six fluffy loci were initially identified that were the result of recessive mutations in the *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE* genes. Fluffy mutants have similar phenotypes in being deficient in conidiophore formation and *brlA* expression (4, 5, 37, 70, 123). Two of these genes, *fluG* and *flbA*, were later found to regulate both asexual development and sterigmatocystin production (55), with *fluG* believed to act upstream of *flbA* (Fig. 3). *fluG* is involved in the synthesis of an extracellular low-molecular-weight diffusible factor that activates a pathway leading to conidiation and sterigmatocystin production (69). *FluG* loss-of-function mutations

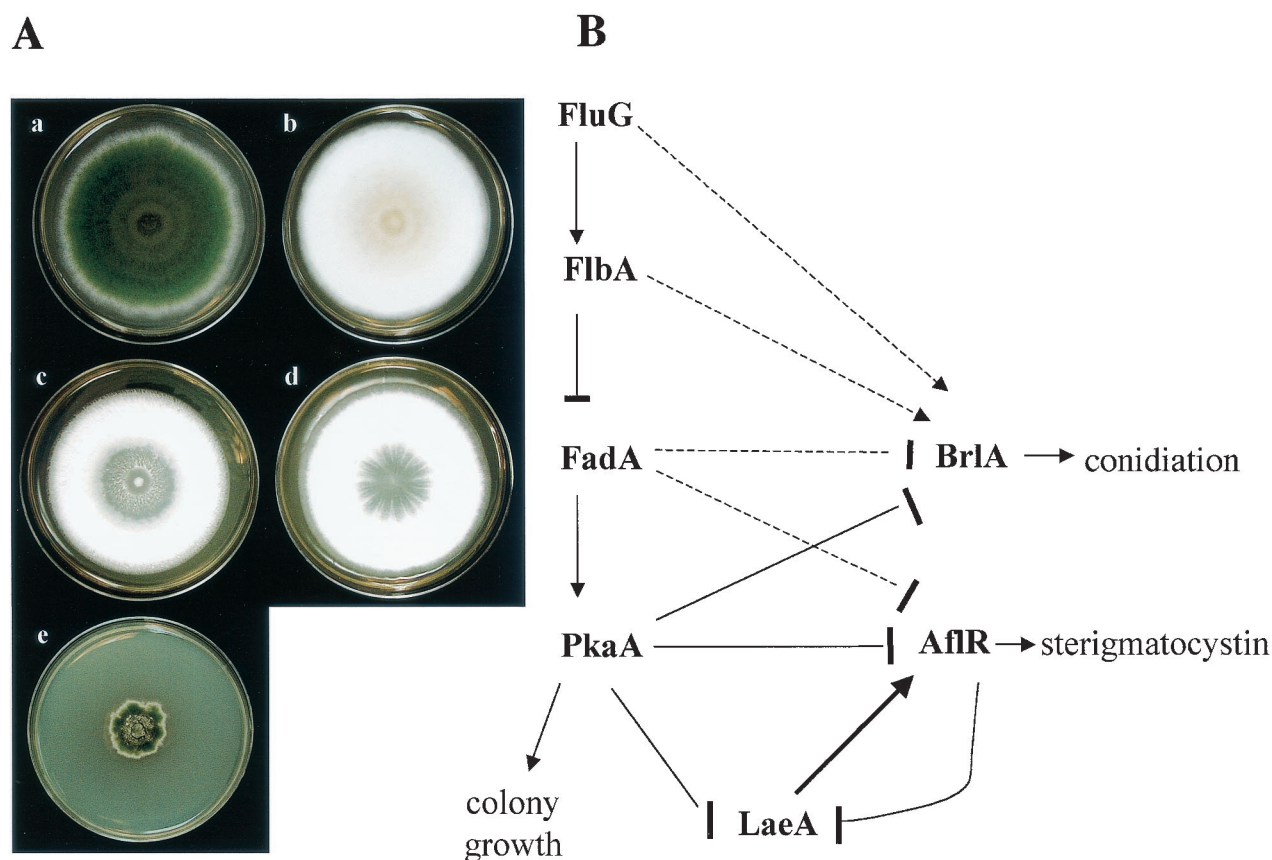


FIG. 3. (A) Phenotype effect of signaling mutants. Strains shown are wild-type *A. nidulans* (a), $\Delta fluG$ (b), $\Delta flbA$ (c), $fadA^{G42R}$ (d), and $\Delta pkaA$ (e). (B) Proposed model of G-protein signal transduction regulating sterigmatocystin production and sporulation. Arrows indicate positive regulation, and blocked arrows indicate negative regulation. Solid lines indicate known pathways. Dashed lines indicate postulated pathways. Abbreviations: FluG, early acting developmental regulator; FlbA, regulator of G-protein signaling; FadA, α subunit of G-protein; PkaA, catalytic subunit of PKA; LaeA, AflR regulator; AflR, sterigmatocystin-aflatoxin-specific transcription factor; BrlA, conidiation-specific transcription factor (55, 106).

(e.g., $\Delta fluG$, Table 2 and Fig. 3) present an aconidial, non-sterigmatocystin-producing phenotype (55). Overexpression of *fluG* in submerged culture was sufficient to induce *brlA* expression and subsequent conidiation (68). However, *fluG* overexpression was not sufficient to induce precocious sterigmatocystin production, indicating that FluG-independent regulatory elements are required in sterigmatocystin gene cluster activation (55).

FlbA loss-of-function mutants exhibit a phenotype similar to those of $\Delta fluG$ strains, and in addition, the mycelium starts to autolyze after several days of growth (55) (Table 2 and Fig. 3). The FlbA protein was found to contain a regulator of G-protein signaling (RGS) domain (70), suggesting that $\Delta flbA$ strains harbor a constitutively active G-signaling pathway responsible for the aconidial, non-sterigmatocystin-producing phenotype (Fig. 3). This was supported by the identification of another mutant, $fadA^{G42R}$, presenting the same phenotype as $\Delta flbA$. *fadA* is an α subunit of a heterotrimeric G protein, putatively the target for the RGS domain of *flbA* (133). When bound to GTP, FadA is in an active form that favors vegetative growth (Fig. 3). Developmental activation leading to sporulation and sterigmatocystin production presumably requires FlbA to enhance the intrinsic GTPase activity of FadA. The $fadA^{G42R}$ mutant is a dominant-activated form of FadA unable

to hydrolyze GTP (55) (Table 2 and Fig. 3). Mutants overexpressing *flbA* or mutants carrying a *fadA* dominant negative allele where FadA is inactive, e.g., $fadA^{G203R}$, show premature sterigmatocystin production and conidiation (55). These results support a model in which FlbA negatively regulates the FadA signaling pathway that blocks conidiation and sterigmatocystin biosynthesis (Fig. 3).

Loss-of-function *flbA* or $fadA^{G42R}$ dominant activating mutations block sterigmatocystin production and conidiation through inhibition of the expression of the sterigmatocystin gene transcription factor *aflR* and the asexual developmental transcription factor *brlA* (55, 133). Conidiation can be rescued in loss-of-function *flbA* mutants by forced *brlA* overexpression (J. Hicks and N. P. Keller, unpublished data). However, *aflR* overexpression failed to reestablish sterigmatocystin production in a $\Delta flbA$ background (K. Shimizu, J. Hicks, and N. P. Keller, unpublished data). These results suggest differential downstream regulation in the signal transduction pathway for *brlA* and *aflR*.

Role of *pkaA*, which encodes a PKA catalytic subunit. The understanding of the mechanism by which FlbA and FadA regulate conidiation and sterigmatocystin production was recently extended by characterization of a cAMP-dependent protein kinase catalytic subunit (*pkaA*) (106). Deletion of *pkaA*

TABLE 2. Alleles having effects on sporulation and/or sterigmatocystin production.

Allele(s)	Function	Mutation effect	Reference(s)
<i>afIR</i>	Pathway-specific regulator of <i>stc</i> gene expression		91
$\Delta afIR$		Loss of sterigmatocystin-aflatoxin production	29, 92, 131
<i>OEafIR</i>		Affects conidiation and sclerotial development	25, 97, 108, 120
		Early and increased mycotoxin production	106
<i>brlA</i>	Pathway-specific regulator of conidiogenesis genes		31
$\Delta brlA$		Abolishment of conidiation	17, 58
<i>OEbrlA</i>		Early onset of conidiation, including sporulation in submerged liquid culture; no effect on sterigmatocystin production	8
<i>fluG</i>	Involved in synthesis of extracellular, diffusible factor required for conidiation		69
$\Delta fluG$		Aconidial; no sterigmatocystin production	55
<i>OEfluG</i>		Activates conidiation; early sterigmatocystin production observed in submerged liquid culture but not on plates	55
<i>flbA</i>	Encodes RGS-containing protein; active FlbA inactivates G-protein pathway		70
$\Delta flbA$		Aconidial; no sterigmatocystin production	55
<i>OEflbA</i>		Premature sporulation, early sterigmatocystin production	55
<i>fadA</i>	α -Subunit of heterotrimeric G protein		133
$\Delta fadA$ and <i>fadA</i> ^{G203R}		Premature conidiation; early sterigmatocystin production	55
<i>fadA</i> ^{G42R}		Aconidial; no sterigmatocystin production	55
<i>pkaA</i>	cAMP-dependent PKA		106
$\Delta pkaA$		Results in hypersporulation; aberrant sterigmatocystin production	106
<i>OE pkaA</i>		Decreased conidiation; no sterigmatocystin production	106
$\Delta pkaA \Delta flbA$		Double deletion mutant; partially restores some sporulation; restores sterigmatocystin production	106
$\Delta pkaA fadAG42R$		Partially restores sporulation but not sterigmatocystin production	106
<i>sfaD</i>	β -Subunit of heterotrimeric G-protein		99
$\Delta sfaD$		Increased sporulation	105

^a Δ indicates the gene has been deleted. OE indicates the gene is overexpressed. *fadA*^{G203R} is the dominant negative *fadA* allele. *fadA*^{G42R} is the dominant activated *fadA* allele.

leads to hyperconidiation and has little effect on sterigmatocystin production, while *pkaA* overexpression inhibits *brlA* and *afIR* expression and, concomitantly, conidiation and mycotoxin production—the latter being a phenotype resembling $\Delta flbA$ and dominant-activated *fadA*^{G42R} mutants (106) (Table 2 and Fig. 3). Deletion of *pkaA* restores conidiation in the $\Delta flbA$ and *fadA*^{G42R} backgrounds but restores sterigmatocystin production only in the $\Delta flbA$ but not *fadA*^{G42R} background (106). These data suggest that FlbA and FadA inhibition of conidiation is mediated in part through PkaA and that FlbA has a FadA-independent, PkaA-dependent role in regulating sterigmatocystin production. In addition to transcriptionally regulating *brlA* and *afIR*, PkaA also regulates AfIR and perhaps BrIA posttranscriptionally (106). Recent studies suggest that PkaA negatively regulates AfIR activity through phosphorylation (J. Hicks, K. Shimizu, and N. P. Keller, Abstr. XXI Fungal Genet. Conf., p. 144, 2001). Transcriptional regulation of AfIR by

PkaA might be mediated by LaeA, a protein required for *afIR* expression, and negatively regulated by PkaA (J. W. Bok and N. P. Keller, Abstr. XXI Fungal Genet. Conf., p. 144, 2001) (Fig. 3).

Other Components of the Signal Transduction Pathway Controlling *A. nidulans* Development

With regards to *Aspergillus* development, several other signaling proteins have been identified that are involved in sporulation and colony formation. One protein, RasA, is a homolog of the yeast Ras proteins. The levels of activated RasA protein in fungal cells regulate the development of *A. nidulans* (111). High levels of active RasA result in spores that cannot produce germ tubes, whereas intermediate levels lead to uncontrolled vegetative growth similar to the $\Delta flbA$, *fadA*^{G42R}, and *pkaA* overexpression phenotypes. Preliminary data suggest that ac-

tivated Ras protein also inhibits sterigmatocystin production (K. Shimizu and N. P. Keller, unpublished data). In *S. cerevisiae* but not *S. pombe*, Ras activity is partially mediated through PKA, and efforts are under way to determine if Ras inhibition of development and sterigmatocystin production in *A. nidulans* requires PkaA activity.

Recently, Fillinger et al. (43) isolated *cyaA*, which encodes adenylate cyclase, from *A. nidulans* (43). Deletion of *cyaA* severely affects spore germination, causing a delay in germ tube emergence, but the effect on sterigmatocystin production is unknown.

The *sfaD* gene of *A. nidulans* encodes the β -subunit of the heterotrimeric G-protein complex (99). *sfaD* deletion mutants exhibit conidiation in submerged cultures, similar to the phenotype described for strains containing a dominant negative allele of *fadA* (i.e., *fadA*^{G203R}), but unlike in *fadA*^{G203R} strains, sterigmatocystin production is reduced in the Δ *sfaD* mutant under these conditions (Shimizu and Keller, unpublished) (Table 2). In addition, constitutive active FadA can cause proliferative growth in the absence of *sfaD*. Also, introduction of Δ *sfaD* into a Δ *flbA* background can restore sporulation (99, 132). These results suggest that SfaD and FadA are both positive growth regulators with partially overlapping functions and that FlbA controls both proteins.

Conservation of FadA Regulation of Aflatoxin Production in Other *Aspergillus* spp.

Proteins of the G-protein signaling pathway of *A. nidulans* appear to have functional counterparts in *A. parasiticus* and *A. flavus*. Hicks et al. (55) transformed the *A. nidulans* *fadA*^{G42R} dominant active allele into a norsolorinic acid (e.g., the first stable intermediate in the aflatoxin pathway)-accumulating strain of *A. parasiticus*. The resultant strain did not produce norsolorinic acid and had the same fluffy phenotype as an *A. nidulans* *fadA*^{G42R} mutant (55). S. C. Sim and N. P. Keller (unpublished data) obtained an aconidial, null aflatoxin phenotype in *A. flavus* strains transformed with the *fadA*^{G42R} allele.

Signal Transduction Regulation of Sporulation and Secondary Metabolism in Other Organisms

Antibiotics. The best-known example of a genetic connection between antibiotic production and morphological development is found in the filamentous bacteria *Streptomyces* spp., in which common elements regulating sporulation and antibiotic production have been reported (56). For example, the translation regulator *bldA* (67) encodes a tRNA essential to translate the ATT leucine codon, which is scarce in *Streptomyces*. Deletion of this gene results in reduction of spore production and failure to synthesize antibiotics, without affecting growth. In *A. nidulans*, a relationship between conidiation and the production of the antibiotic penicillin has also recently been described (115). The dominant activating *fadA*^{G42R} allele, which represses conidiation and sterigmatocystin biosynthesis, stimulates the expression of *ipnA* (an enzymatic gene of the *A. nidulans* penicillin gene cluster) and stimulates concomitant penicillin biosynthesis (115).

Cyclopiazonic acid. Several strains of *A. flavus* produce the mycotoxin cyclopiazonic acid as well as aflatoxin. Introduction of the *fadA*^{G42R} allele into *A. flavus* inhibits the production of both mycotoxins (Sim and Keller, unpublished).

Trichothecenes. Introduction of the *A. nidulans* *fadA*^{G42R} allele into the fungus *F. sporotrichioides* generated strains with reduced conidial production and increased trichothecene production (115). Expression of the *fadA*^{G42R} allele reduced *Fusarium* spore production 50 to 95% and resulted in delayed radial growth compared with the wild type.

Pigments. In *Cryphonectria parasitica*, the chestnut-blight fungus, deletion of *cpg1*, which encodes the α -subunit of a heterotrimeric G protein, leads to a marked reduction in fungal growth rate and spore production and a loss of virulence. Reduced pigmentation is also observed (45). The β -subunit of a G protein, Cpg2, has also been identified in this fungus. Its deletion also decreases virulence, conidiation, and pigment production (62).

OTHER GENETIC FACTORS COUPLING DEVELOPMENT AND SECONDARY METABOLISM

CCAAT Binding Protein Complex

The *A. nidulans* CCAAT binding protein complex PENR1 has been reported to regulate both development and penicillin production (74, 112). PENR1 positively regulates the expression of two penicillin biosynthetic genes, *ipnA* and *aatA*. Deletion of *hapB*, *hapC*, and *hapE* genes, which encode PENR1 complex components, resulted in identical phenotypes characterized by low levels of *ipnA* and *aatA* expression as well as a slow-growth phenotype and a reduction in conidiation (74, 87, 112).

WD Protein Regulation

A. nidulans *rcoA* encodes a member of the WD protein family. These proteins contain WD repeats, conserved sequence motifs usually ending with Trp-Asp (WD) (110). Members of this family have a role in signal transduction pathways and/or nuclear scaffolding complexes and/or act as global regulators involved in repression of a broad number of genes (84, 122). Several WD proteins have been found to be important in fungal development. In *S. pombe*, Frz1 is required for spore production (9). In *Neurospora crassa*, RCO1 is involved in the control of vegetative growth, sexual reproduction, and asexual development (128). *S. cerevisiae* Tup1p is the best-known example of a fungal WD global regulator. Tup1p forms a complex with other proteins and interacts with histone H3 and H4, stabilizing the nucleosomes on the gene promoters and hence blocking access by the RNA polymerase II complex (38).

RcoA in *A. nidulans* (53) has closest similarity to *N. crassa* RCO1, and the respective mutants share some similar aberrance in growth. *A. nidulans* Δ *rcoA* strains are deficient in both sterigmatocystin and asexual spore production (53). *brlA* and *aflR* transcription is greatly suppressed in these strains, but *fluG*, *flbA*, and *fadA* expression is not affected, thus suggesting that the regulatory role of RcoA may be independent of the G-protein signaling pathway.

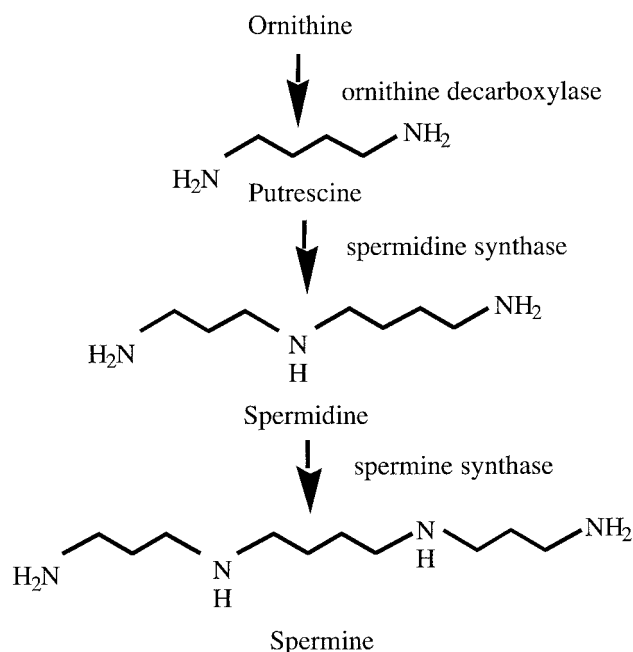


FIG. 4. Polyamine biosynthetic pathway.

Polyamine Pathway

Polyamines are small aliphatic molecules important for normal cell growth and development in a wide range of organisms (100). The most common polyamines are spermidine, spermine, and putrescine, and their biosynthetic relationship is shown in Fig. 4. Rajam and Galston (96) showed that inhibitors of polyamine biosynthesis reduced mycelial growth when applied to cultures of some phytopathogenic fungi, demonstrating the importance of these molecules for fungal development. Guzman-de-Peña et al. (49) described a positive correlation between polyamine biosynthesis and asexual development, sexual development, and sterigmatocystin production. Diamino butanone, an inhibitor of ornithine decarboxylase (the enzyme required for putrescine biosynthesis), blocked the formation of aerial mycelium, asexual and sexual sporulation, and sterigmatocystin biosynthesis in *A. nidulans* and asexual sporulation and aflatoxin biosynthesis in *A. parasiticus* (50). Supplementation of exogenous putrescine could partially overcome the repressive effects of the drug. In *A. nidulans*, spermidine is essential for growth, sporulation, and mycotoxin production, since deletion of the *spdA* gene, which encodes a spermidine synthase, is lethal (J. Yuan, J. W. Bok, D. Guzman-de-Peña, and N. P. Keller, Mol. Microbiol., in press). Supplementing media with spermidine restores growth and allows asexual sporulation and sterigmatocystin production to resume (Jin et al., submitted).

pacC

In an earlier section we stated that pH affected fungal development and sterigmatocystin and aflatoxin production in *Aspergillus* spp. in a complex fashion. Keller et al. (65) hypothesized that control of aflatoxin and sterigmatocystin gene expression in response to pH could be mediated via the global

transcriptional regulator PacC (118). At acidic pHs, the PacC protein is in an inactive conformation and is unable to bind target sites. At alkaline pHs, the PacC protein is cleaved (40), producing an active form. This activated PacC isoform can then bind to the promoters of target genes, activating the expression of alkaline expressed genes, and repressing the expression of acid expressed genes. This suggested that PacC might be a negative regulator of aflatoxin-sterigmatocystin gene expression, as these genes were more highly expressed at acidic pH (65). Ehrlich et al. (39) showed that the promoter region of the *A. parasiticus aflR* contains at least one PacC binding site and several promoters of *stc* genes in *A. nidulans* also contain putative PacC binding sites (65), thus allowing for the possibility of aflatoxin-sterigmatocystin regulation by PacC. Besides possibly regulating aflatoxin-sterigmatocystin production (65), PacC is also a regulator of genes involved in penicillin production (41, 118). At elevated pHs, PacC activates expression of the isopenicillin N synthase gene, which is involved in penicillin biosynthesis. In *Penicillium chrysogenum*, PacC also activates the expression of the isopenicillin N synthase homologue, *pcbC* (114). In addition, PacC regulates morphogenesis. Deletion of the entire *pacC* coding region results in reduction of growth and conidiation (118). Truncated *pacC* alleles are also deficient in conidiation (94).

areA

As previously mentioned, Cotty (32) studied the influence of the nitrogen source on development and aflatoxin production in *A. flavus* and determined a link between nitrogen source, aflatoxin production, and sclerotial morphogenesis. The influence of nitrogen source on sclerotial production at the molecular level is still unclear. However, the repression of aflatoxin production by nitrate might be partially explained by AreA-mediated regulation. AreA is a positive-acting repressor of genes involved in nitrogen metabolism. This GATA factor recognizes specific sequences in the promoter regions of genes under its control (124). *areA* has been recently cloned in *A. parasiticus*, and it has been shown in electrophoretic mobility shift assays that AreA binds the region between *aflR* and *aflJ* in this fungus (30). This finding suggests that *areA* participates in the nitrate-mediated negative regulation of gene transcription for aflatoxin biosynthesis in *A. parasiticus*. Liu and Chu (75) showed that *aflR* expression in *A. parasiticus* was inhibited by nitrate. This inhibition can be overcome by overexpression of *aflR* (28). Nitrate might also regulate aflatoxin biosynthesis posttranscriptionally. Flaherty and Payne (44) overexpressed *aflR* in nitrate and media lacking nitrate. Although aflatoxin transcripts were detected in both cases, aflatoxin biosynthesis inhibition in the presence of nitrate was not alleviated. This result indicates that nitrate regulates mycotoxin production not only transcriptionally but also posttranscriptionally.

C-Type Cyclins

Fumonisin is a group of mycotoxins produced by *F. verticillioides*. One of the genes involved in fumonisin biosynthesis, *FUM5*, encodes a polyketide synthase and has recently been characterized (95). Recently, evidence connecting fumonisin production and sporulation has been reported (104). A regu-

lator gene of the fumonisins pathway has been found, *FCC1*, which encodes a putative polypeptide with similarities to C-type cyclins of *S. cerevisiae* (73, 104). Cyclins are essential activating subunits of cyclin-dependent kinases and include a large family of proteins with conserved function in many organisms (7). The *FCC1* mutant does not express *FUM5* and therefore does not produce fumonisin B₁. Additionally, the *FCC1* mutant shows reduced asexual spore production when grown on cracked corn. However, the effect of *FCC1* mutation on fumonisin production and development was shown to be pH dependent, as the wild-type phenotype was partially recovered in low-pH media.

CONCLUSIONS

It has been noted since the earliest days of fungal manipulation that many species of filamentous fungi readily synthesize complex compounds that are putatively helpful but not necessary for survival and whose production is presumably costly to maintain. Furthermore, production is often linked to fungal development. Some compounds might function as virulence factors, or their presence could give a competitive edge to the producing organism or enhance the survivability of spores. Some secondary metabolites stimulate sporulation and therefore influence the development of the producing organism and neighboring members of the same species, perhaps enhancing the fitness of a community of related species. Natural products are often produced late in fungal development, and their biosynthesis is complex. This complexity is due to a number of factors that affect secondary metabolite production. These include (i) the influence of a number of external and internal factors on natural product biosynthesis, (ii) the involvement of many sequential enzymatic reactions required for converting primary building blocks into natural products, (iii) tight regulation of natural product enzymatic gene expression by one or more transcriptional activators, (iv) close association of natural product biosynthesis with primary metabolism, and (v) close association of natural products with later stages of fungal development, particularly sporulation. Furthermore, the genes required for biosynthesis of some natural products are clustered, perhaps as a consequence of these factors. Gene clusters contain all or most of the genes required for natural product biosynthesis, and logic suggests that their maintenance could only be selected for if the final natural product conferred some advantage to the producing organism. Moreover, natural product biosynthetic gene clusters can be conserved between organisms, for example the sterigmatocystin-aflatoxin biosynthetic gene cluster in several *Aspergillus* spp.

As discussed in the preceding sections, some natural products directly enhance sporulation of the producing organisms. Relatively few of these compounds have been described. Other secondary metabolites function to protect the spore; for example, melanin is a protectant from UV damage. Most natural products play no obvious roles in sporulation or spore protection but are secreted into the environment at a time in the life cycle of the fungus that corresponds with sporulation. However, some of the compounds that are excreted into the environment could have subtle effects on the organism that are not immediately obvious. For example, although sterigmatocystin was long thought to have no effect on the producing fungus,

recent studies (97, 108) have demonstrated an effect of sterigmatocystin on *Aspergillus* spore production.

Aspergillus spp. produce mycotoxins at a time that coincides with spore development. In *A. nidulans*, these processes are regulated by G-protein signaling pathway components (55, 106, 133). Inactivation of a G α -subunit, *FadA*, leads to concomitant sporulation and mycotoxin production. Some of the G-protein signaling regulatory elements described for *A. nidulans* are conserved among other filamentous fungi (6, 16, 55, 62, 66, 115, 116, 129). However, they can have different or even opposite roles in regulating the biosynthesis of natural products (115). As Tag and collaborators reported, the *fadA*^{G42R} allele negatively regulates sterigmatocystin production but positively regulates trichothecene and penicillin production (115). Why should the same G-protein signaling pathway oppositely regulate two or more different natural products? The particular example of opposite regulation of sterigmatocystin and penicillin production in *A. nidulans* by the same signaling pathway allows us to speculate on the necessity of differential regulation when we consider that these two metabolites are also oppositely regulated by pH (penicillin is favored in alkali environments, and sterigmatocystin is favored in acidic environments). Although penicillin is destroyed at low pH, aflatoxin and sterigmatocystin are very stable molecules not affected by pH extremes. All three of these molecules are complex and incur a large energy cost. Aflatoxin is known to be toxic to insects (90), while penicillin is a renowned bactericidal antibiotic. One could speculate that in their natural environment, *Aspergillus* spp. face more competition from bacteria in alkaline soils, while acidic soils are generally less populous in bacteria. Therefore, penicillin is produced during fungal development in alkaline environments to destroy its bacterial competition, while sterigmatocystin and aflatoxin secretion and accumulation in mycelial and sclerotial tissue in acidic environments provide protection against insects. Penicillin and sterigmatocystin biosynthesis is temporal in nature, with penicillin biosynthetic gene transcription occurring earlier than *stc* gene transcription (115). This would ensure that penicillin is secreted into the environment first to kill off fast-growing prokaryotes, allowing the fungus to establish itself in the community. Later, penicillin production ceases and sterigmatocystin is produced to protect against eukaryotic competitors.

The G-protein signaling pathway operates in most organisms and appears to be a conserved mechanism linking external stimuli to a coordinated response by the organism. In *Aspergillus*, homologs of familiar proteins identified in other organisms, such as an RGS domain protein, α - and β -subunits of heterotrimeric G proteins, and cAMP-dependent PKA, are involved in controlling genes involved in conidiation (via *brlA* expression) and mycotoxin production (via *aflR* expression). Although most of the work on the G-protein signaling pathway linking mycotoxin production and sporulation has been done using *Aspergillus* species, particularly *A. nidulans*, evidence indicates the presence of a similar pathway regulating trichothecene production in *Fusarium* (115). The G-protein signaling pathway is an appealing mechanism to link fungal development with natural product metabolism for the following reasons: environmental influences on sporulation and sterigmatocystin and aflatoxin production in *Aspergillus* spp. can be very complex and contradictory, particularly with regards to pH. If mul-

multiple signals for development and mycotoxin production are received external to the cell by different receptors that sense pH, sugar, and nitrogen content of the environment, a system is required to integrate these signals and communicate a coherent message to the nucleus, leading to a coordinated response. Because of the ubiquitous nature of the G-protein signaling pathway in higher organisms, it seems likely that *Aspergillus* has coopted this pathway to link sporulation and development with mycotoxin production. We speculate that the same mechanism could operate in other fungi that excrete natural products at the onset of sporulation. However, this signaling pathway might not be the only mechanism to coordinate natural product biosynthesis and sporulation. In liquid shake cultures, *Aspergillus* does not sporulate but still produces mycotoxins. Furthermore, some linoleic acid-derived seed compounds can promote sporulation but inhibit aflatoxin gene transcription as described below.

Aspergillus spp. produce mycotoxins during the onset of sporulation. These compounds might influence the fitness of the producing organism but are not considered sporulating factors per se. However, this genus also secretes linoleic acid-derived molecules, termed psi factors, which do influence asexual and sexual development of the fungus (27). In addition, some plant unsaturated fatty acids have been shown to influence asexual and sexual development (24) and mycotoxin production (22) in this fungus. It is interesting to speculate why *Aspergillus* spp. produce sporulating molecules that resemble host seed constituents. Perhaps the production of secondary metabolites that mimic the effect of oilseed compounds on fungal growth and development represents a mechanism for survival by the fungi when it is not feeding on a host seed. For *A. nidulans*, a soil organism, the production of linoleic acid-derived psi factors required for development could indicate it evolved from oilseed-infesting aspergilli.

Aflatoxin and sterigmatocystin production in *Aspergillus* spp. has been extensively studied due to the deleterious effect of the mycotoxin on human health. While medical investigations have focused on understanding the mechanisms of aflatoxin toxicity and carcinogenicity in humans, these effects of sterigmatocystin and aflatoxin on human health are a consequence of ingesting infected crops and are not the primary function of these compounds. Why these fungi produce sterigmatocystin and aflatoxin; what effect, if any, these compounds have on fungal development; and what ecological role the mycotoxins play are complex questions. In *A. nidulans*, mutants that produce no sterigmatocystin or accumulate different intermediates of this mycotoxin have been shown in corn and medium-based experiments to be less fit than wild-type strains (97, 108), as defined by reduced sporulation. The further one proceeds along the sterigmatocystin pathway, the more fit the organism becomes. Therefore, these results suggest sterigmatocystin can increase the competitive fitness of the organism.

In recent years, great progress has been made in the discovery of signaling pathways that connect fungal development with natural product biosynthesis. However, much remains to be learned. The complexity of these regulatory networks, with multiple target sites and interconnections with other regulatory mechanisms, makes their full elucidation a challenging task. Emerging technologies should be brought to bear on this puzzle. *A. nidulans* has a large number of mutants available to

address these issues. Furthermore, most of the *A. nidulans* genome has been sequenced and is available (Cereon Inc.). An illuminating set of experiments could involve microarray analysis of genes involved in the G-protein signaling pathway, fatty acid metabolism (as unsaturated fatty acids influence development), acetate metabolism (acetate is derived from the catabolism of fatty acids and is the primary building block for aflatoxin and sterigmatocystin), and sterigmatocystin biosynthesis. Information obtained from the model organism *A. nidulans* could lead to an understanding of how these regulatory pathways directly or indirectly control fungal development and the biosynthesis of natural products in other microorganisms. This will open up new broad and exciting fields of applications in which the production of beneficial natural products could be enhanced and the production of those with deleterious effects could be reduced or eliminated.

ACKNOWLEDGMENTS

A. M. Calvo and R. A. Wilson made equal contributions to this work.

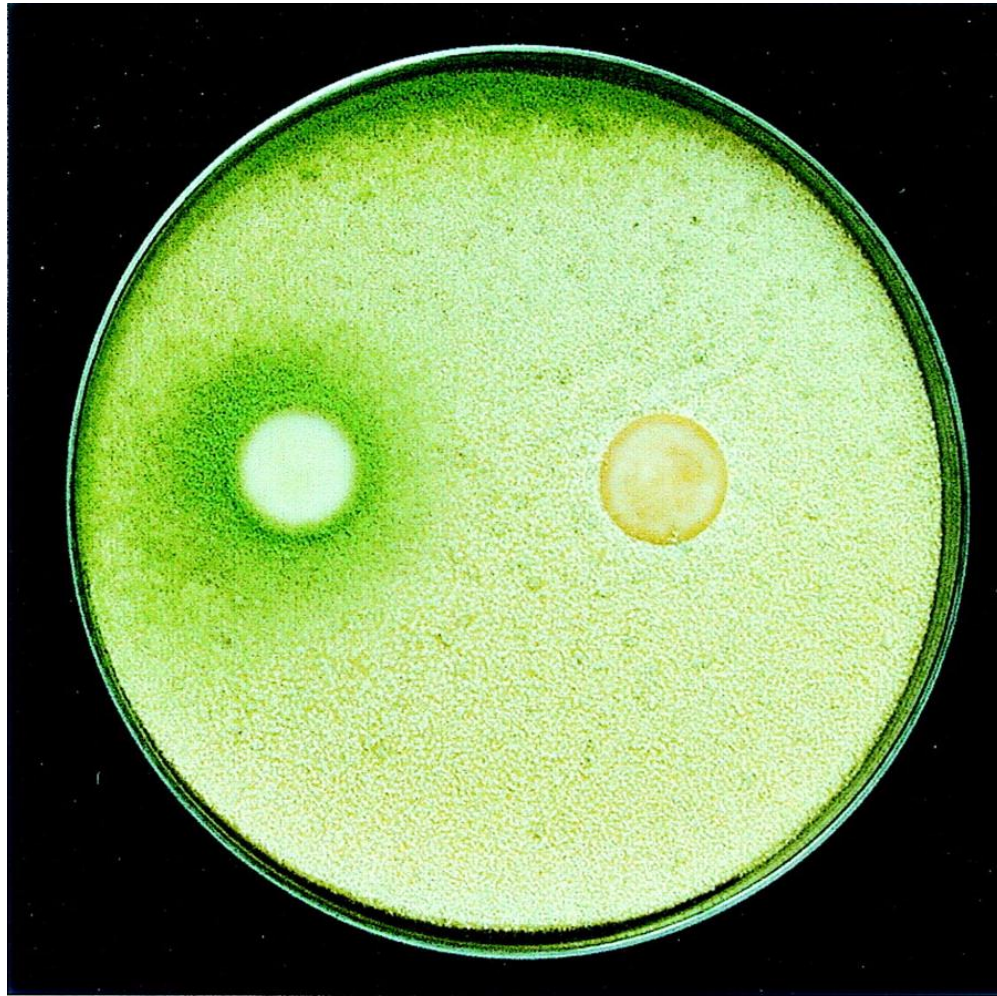
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Sporogenic effect of linoleic acid-derived compounds on *Aspergillus*.



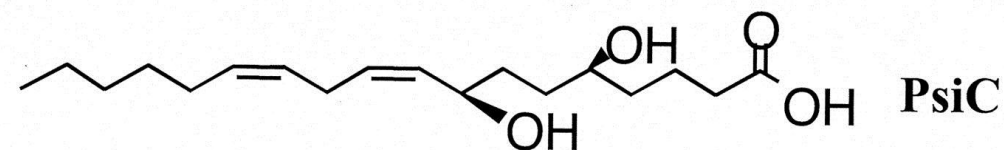
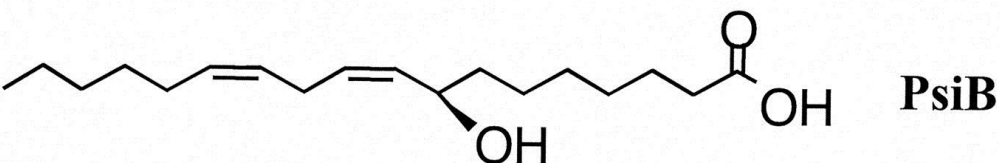
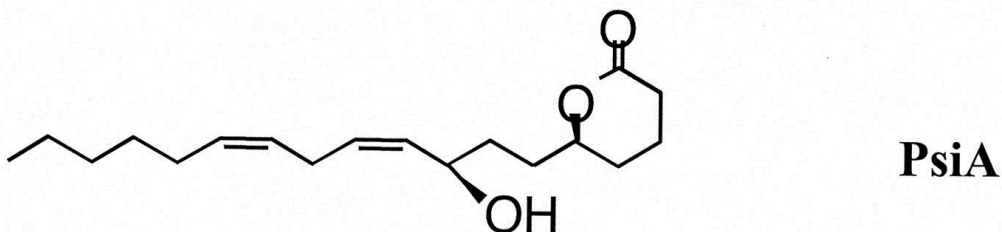
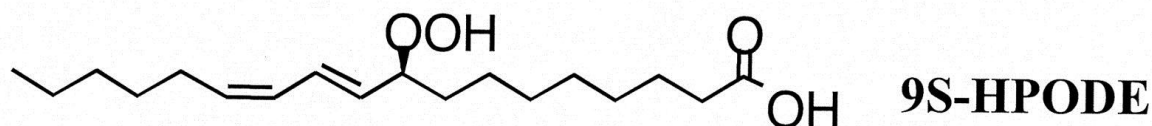
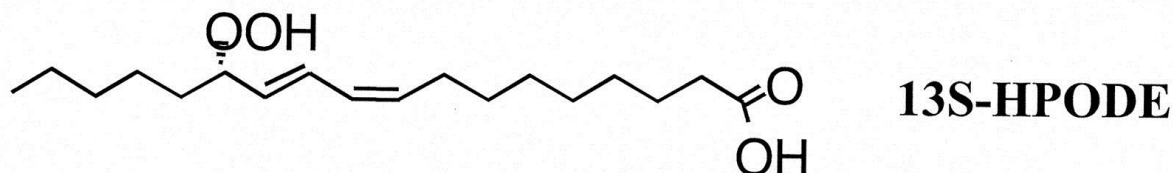
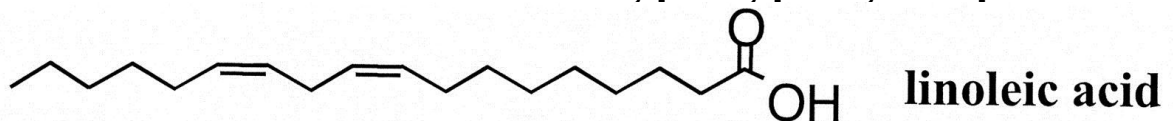
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Structures of linoleic acid and linoleic acid-derived molecules from seed-, 13S-hydroperoxylinoleic acid (13S-HPODE)- and 9S-HPODE-, and linoleic acid-derived sporogenic molecules from *A. nidulans*, psiA, psiB, and psiC.

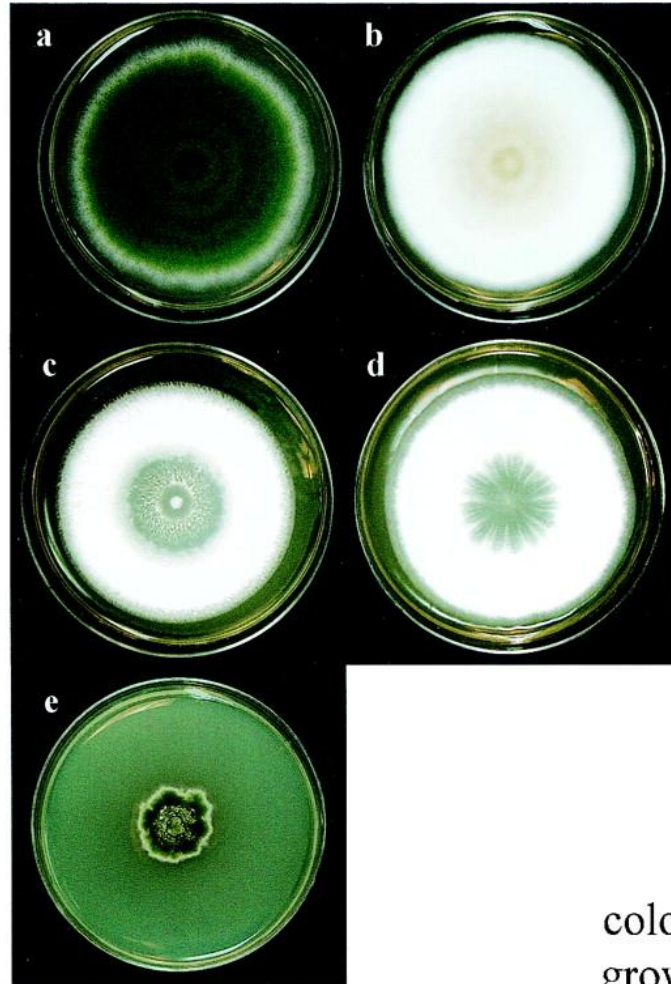


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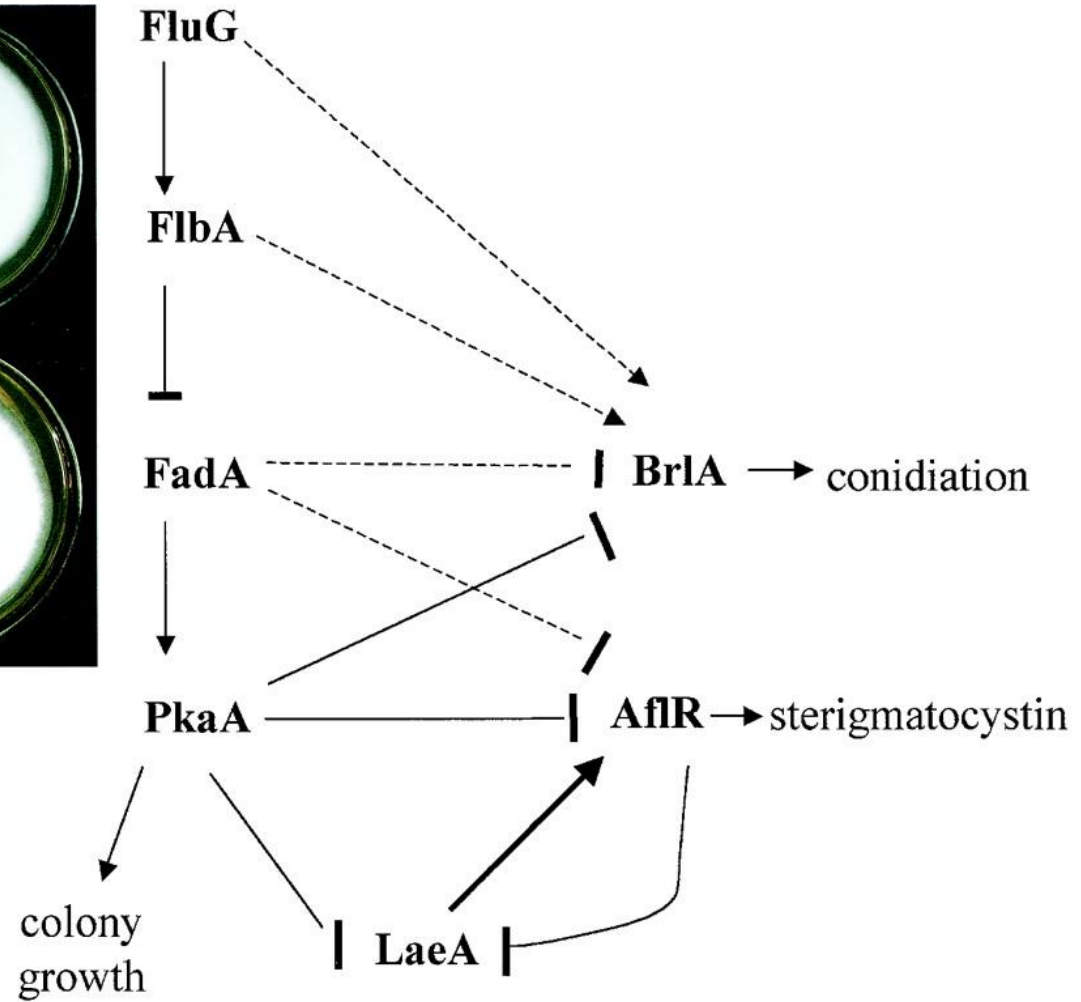
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(A) Phenotype effect of signaling mutants.

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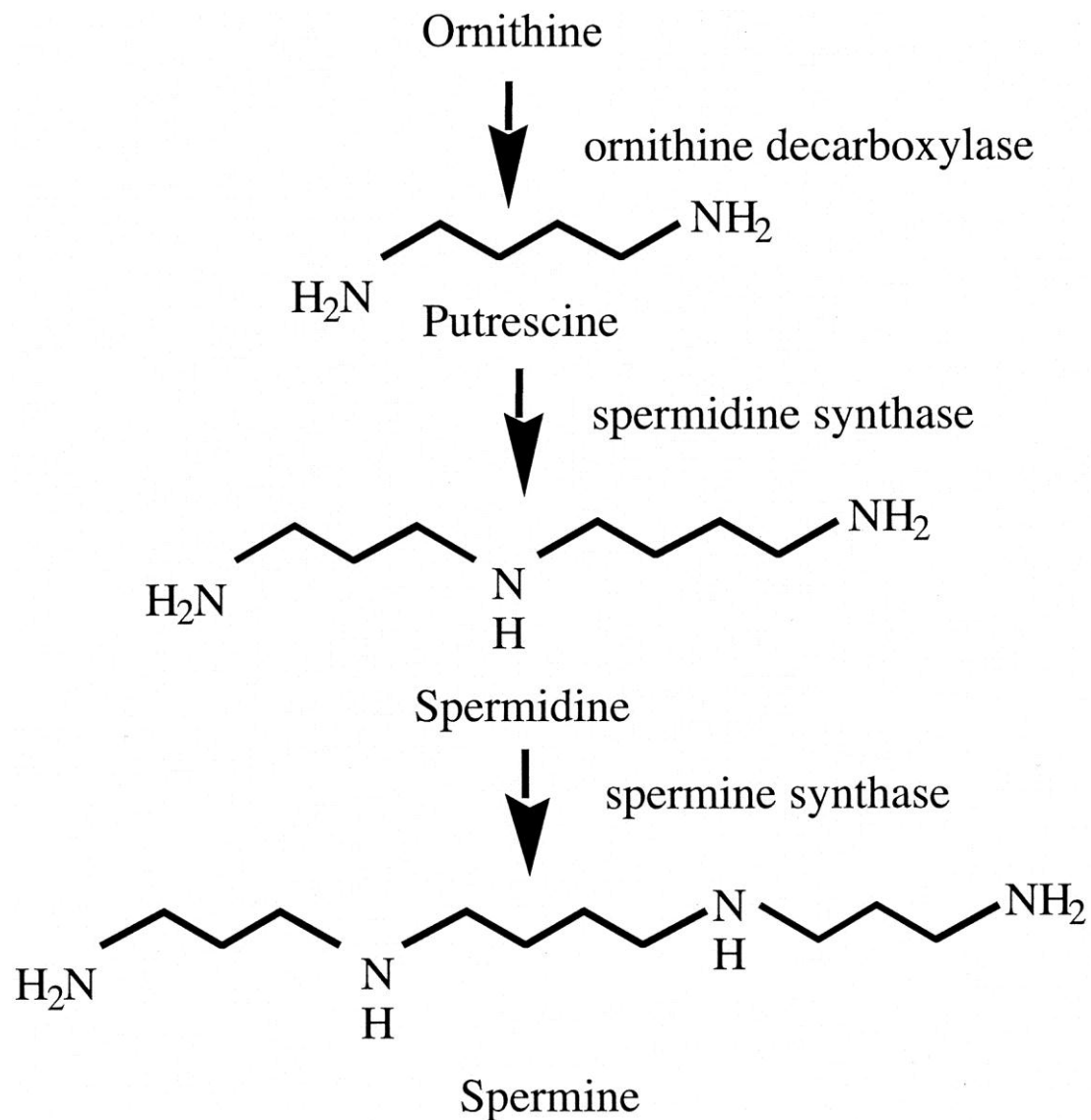
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